FINAL REPORT

PROJECT B-276

BACTERIAL RESPONSE TO CHLORINATED PROTEINS

ROBERT S. INGOLS

Contract No. WP-00188-06

1 September 1965 to 31 August 1966

Prepared for
National Institutes of Health
Washington, D. C.

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BACTERIAL RESPONSE TO CHLORINATED PROTEINS

Chlorination of water supplies has provided the consumer with excellent protection against pathogenic bacteria. Chlorination of raw waste water has been practiced successfully for reduction of either odor in pipelines or the BOD during low river flows and/or pathogenic bacteria. Only small concentrations of chlorine are used in potable water treatment, because the organic matter concentration must be low to be a suitable supply. The organic matter concentration in waste water, however, is much higher than in the raw water (by as much as 100 fold). Therefore, the chlorine demand is much higher and large quantities may be used per day in only partial fulfillment of the demand.

Much of the chlorine added either to water or waste water will be reduced to chloride ions while oxidizing the organic and inorganic reductants in the water. A part of the chlorine, however, which is added to waste water will substitute on the organic matter. The chlorine reduced to the chloride ion will produce new compounds with lower biochemical oxygen demand, but with no residual toxic effects (6). The chlorine substituted on the organic matter is the principal concern of this report because of its possible immediate toxic reaction and of its long term ecological damage in a river system.

Previous work (4) done on this project has shown that while halogenated phenols are toxic the bacteria can degrade them slowly in the absence of other normal food. Phenols as such are not found commonly in nature, but are found as the amino acid, tyrosine (a substituted phenol) in all living protoplasm. Very little is known concerning how much halogenated tyrosine can be incorporated into bacterial protoplasm when it is a part of the food in its environment. A halogenated derivative of tyrosine, thyroxin is a normal constituent

in trace quantities in the hormone of higher animals but the hormone is very toxic in higher than normal concentrations.

This study is considered as a first step in evaluating the effect of substituted chlorine upon bacterial utilization of the chlorinated product. Chlorine can deaminate a free amino nitrogen radical producing a keto acid (7) according to the following equation:

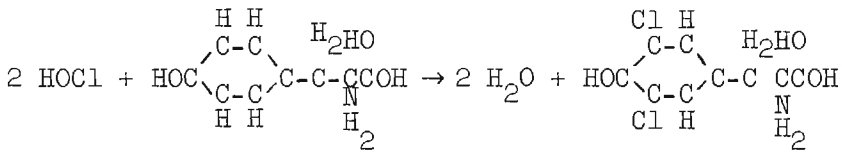
$$2\ CH_3CHNH_2COOH + 5\ HOCl \rightarrow 2\ CH_3COCOOH + 5\ HCl + N_2 + 3\ H_2O \qquad (1)$$

This provides stoichiometric oxidation, but observations (6) have indicated no extra reduction in BOD. The oxidation of a sulfhydryl group may be represented by the following equation (7):

$$RSH + 3\ HOCl \rightarrow RSO_3H + 3\ HCl \qquad (2)$$

This reaction also provides for a stoichiometric reduction in BOD.

The substitution of chlorine on tyrosine can be represented by the following equation:



The reaction is rapid (5). When the tyrosine is free in solution there may also be deamination according to equation #1, but in a protein there will be only a very slow oxidative hydrolysis (7) which requires a considerable excess of chlorine. The reaction of the second equation has been considered by Green and Stumpf (3) as the primary cause of death of bacteria from chlorine. It is the purpose of this study to evaluate whether or not the toxic response of bacteria to chlorinated proteins is sufficient to justify further concern and research interest.

In order to carry out this study, the proteins have been dissolved (dispersed in water), divided into aliquots, sterilized and some aliquots chlorinated. After allowing for complete dissipation of all oxidizing capacity, the various aliquots of the protein solutions have been inoculated and the growth response under various conditions compared with the growth response in an unchlorinated aliquot. Our data indicates that the bacteria respond very slowly to a heavily chlorinated protein as though the environment were bacteriostatic, but not bactericidal. Expressed differently, there is an increasingly lengthened lag period with an increasing ratio of chlorine to protein. Long term effects of the chlorinated protein would also be significant if it can be shown that some of the chlorinated tyrosine is incorporated into the bacterial protoplasm to enter the food chain. If the chlorinated amino acid is incorporated into the food chain, will it stay as part of the protein structure or enter the fat depots of higher animals? This study is considered only the first in a continuing study.

EXPERIMENTAL METHODS

Preliminary investigations were made to determine how much chlorine was needed to produce a residual after 24 hours and no residual after 48 hours. Potassium iodide in acid was used as a qualitative test for residual chlorine. After numerous trials, it was found that 60 mg chlorine per 1.0 g protein could be used to give the required residual. The solution stood for a total of 72 hours before inoculation.

A large volume of protein solution was prepared, divided into flasks suitable for chemical treatment and inoculation, then sterilized. The controls contained added sodium chloride solution to give a constant chlorine content. In general, a control flask and three solutions of chlorinated media were inoculated in each test. The maximum chlorination level was generally 60 mg chlorine per 1.0 g protein, although several fractional doses were also studied. Counts were made at 24 hours and 48 hours. In many runs, aliquots of the suspension were withdrawn for determining changes in turbidity with a Bausch and Lomb Spectronic 20.

Cultures of Escherichia coli and Seratia marcescens were chosen as representatives of organisms with optimum temperatures of 35° and 25°C, respectively. Mixed inocula from sewage were tried, but the varied growth characteristics of the mixed cultures, with clumping either at the cellular or macro level, made the evaluation of the responses very difficult. E. coli and S. marcescens remain as single cells and are well dispersed in an ordinary broth culture for 24 hours.

When sugar was added to the protein solution before sterilizing, the chlorine caused the solution to darken markedly. Apparently, the sugar caramelized, and the color change made it difficult to use turbidity as a

measure of the change in numbers. Counting of viable cells was necessary when this occurred. By adding a sterile sugar solution with the inoculum the darkening was eliminated, and turbidity changes by light transmission could be followed.

In all of the runs shown in Table I, the tryptone and sugar were added, then sterilized. Only a slight darkening occurred upon addition of chlorine, with or without the addition of sugar.

RESULTS

Many individual runs were performed, the tabulated data are averages. The bacterial growth in all chlorinated bacterial media was much less than in the controls (aliquots of the same media to which no hypochlorite had been added).

Table I shows the effect of time and temperature upon the response of *E. coli* and *S. marcescens* to chlorinated nutrient broth medium. The data indicate that at the lowest chlorine dose there was a better response for both species at their optimum temperatures. With each culture after 48 hours, the apparent toxicity drops. Because the control number reaches a maximum at 24 hours, the slow, continuing growth of the organisms in the partially chlorinated media produces a lower toxicity.

One set of growth curves on a multipoint recording turbidimeter indicated an increasing lag, a slower rate of growth, and a slightly lower maximum turbidity with the lowest chlorine concentration.

At the highest chlorine dose, there is a complete retardation in growth, but there is a recovery of the same number of organisms after 24 and 48 hours, indicating that the solution is not bactericidal. It may be considered bacteristatic or the food is simply not available.

Because it is possible that such heavy chlorination destroys some amino nitrogen, some ammonium phosphate was added to one aliquot of the heaviest chlorine does. The growth in each aliquot tested with supplemental nitrogen and phosphate was identical with its control.

Table II shows the effects of adding sterile glucose solution to the medium at the time of inoculation. The chlorine level is 40 mg per 1.0 g protein.

The responses of growth in the chlorinated proteins are compared with the growth in a 2.5 g/l protein concentration, plus sugar at various concentrations. The overall toxicities are much lower than those in Table I with 4.0 g chlorinated protein per liter of medium.

Peptone broth was prepared in 5 concentrations: 1 g/l, 2 g/l, 5 g/l, 10 g/l, 15 g/l. Two groups of these concentrations were prepared, one group chlorinated with 40 mg Cl/g peptone and left for 5 days before inoculating with S. marcescens. Readings of turbidity were taken on the "Spectronic 20". Results as shown in Table III indicate that toxicity of the chlorinated peptone increases with its concentration.

TABLE I

Effects of Time, Temperature and Chlorine Level on Toxicities of Chlorinated Protein. (4.0 g protein and 2 g glucose per liter).

Chlorine level per g protein	Toxicity*							
	E. coli				*S. marcescens*			
	24 hour		48 hour		24 hour		48 hour	
mg/g	22° %	37° %	22° %	37° %	22° %	37° %	22° %	37° %
18	82	40	41	20	66	82	45	71
36	92	27	60	29	95	95	84	87
50	98	99	84	92	99	99	95	96
60	99	90	95	90	94	99	94	86

*Toxicity is expressed as numbers in the sample versus numbers in control as percent.

TABLE II

Effect of Added Glucose

Protein 2.5 g protein/l sugar added	*E. coli* Hours incubation			*S. marcescens* Hours incubation		
grams/per liter	24	48	72	24	48	72
0	--	--	--	5	18	7
1	100	71	48	48	76	59
2.5	18	66	53	82	70	75
5	70	51	58	72	50	71
10	83	35	50	49	--	64

TABLE III

Effect of Concentration of Chlorinated Protein Upon the Toxicity

Chlorinated peptone	Toxicity after growth for 24 hours at room temperature
grams/liter	Percent
1	23
2	47
5	75
10	95
15	98

DISCUSSION

In 1954, Ingols et.al. (7) suggested that the chlorine which had substituted on the tyrosine in the protoplasm of the bacteria might explain part of lethal effect of the hypochlorous acid added to the bacterial suspension. This was vigorously denied by a referee reviewer, but the data of this paper would indicate that even the chlorinated tyrosine in its environment is quite unfavorable to its growth. Theoretically, the bacteria can hydrolyze the protein and use the normal amino acids discarding the chlorinated tyrosine by synthesizing a new chlorine free, tyrosine from the other amino acid residues. It is significant that as the chlorinated protein concentration increases the apparent toxicity increases. Thus, as the controls respond or grow more rapidly there is relatively less use of the chlorinated protein. The presence of sugar could increase the need for all amino acids in the bacterial environment and, thus, be expected to increase the toxicity of the chlorinated protein or to permit the bacteria to more easily synthesize normal amino acids. The data is not clear cut, for both factors may be at work simultaneously under the practical conditions.

From the results of incubation at different temperatures it would appear that bacteria of each specie tested is better able to grow in the presence of the insult at its optimum temperature. Much corollary information is needed to adequately interpret these observations.